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Modulation of the intrahepatic renin–angiotensin system after stimulation of the gastric sodium monitor in the rat

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Key words: angiotensin-converting enzyme (ACE), angiotensinogen, gastric sodium monitor, sodium homoeostasis.

Abbreviations: ACE, angiotensin-converting enzyme; ANG II (etc.), angiotensin II (etc.); VIP, vasoactive intestinal peptide.

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Changes in the rate of formation of angiotensin II (ANG II) participate in mediating the natriuresis that occurs in direct response to a gastric sodium stimulus (upper-gut sodium monitor). As this natriuresis is also dependent on intrahepatic events, we investigated whether changes in hepatic and plasma angiotensinogen levels and hepatic angiotensin-converting enzyme (ACE) activity might explain the decrease in ANG II synthesis. Male Sprague–Dawley rats, equilibrated on a low-sodium diet, were anaesthetized and received a sodium load of 1.5 mmol/kg (using 3× normal saline) either intragastrically or intravenously. Blood and livers were sampled before and at various times after sodium administration. ACE activity in serum and tissues was determined by generation of histidyl-leucine. Angiotensinogen was determined by radioimmunoassay of angiotensin I generated by incubation in the presence of exogenous renin. Plasma angiotensinogen had decreased significantly by 15 min after sodium administration ($P < 0.005$), while hepatic angiotensinogen was also decreased significantly from 30 min after the sodium load ($P < 0.01$). Hepatic ACE activity decreased in response to sodium ($P < 0.005$) from 30 min. We conclude that stimulation of the gastric sodium monitor regulates angiotensinogen synthesis and secretion by the liver, as well as hepatic ACE activity.

INTRODUCTION

The existence of a gastric or portal monitor of sodium intake, which rapidly adjusts urinary sodium excretion in response to dietary sodium intake so that sodium balance is maintained, has been demonstrated in a number of species, including humans [1–4]. It has been postulated that this change in urinary sodium excretion may be mediated neurally. Studies in dogs have suggested that an increase in afferent hepatic nerve activity and a decrease in renal nerve activity [3] participate in the natriuresis that occurs. Other workers have suggested that it may be due to release of humoral mediators from the gastrointestinal or portal tracts [5,6]. One such humoral mediator is the natriuretic peptide vasoactive intestinal peptide (VIP), which is released from the intestine following gastric sodium administration [6]. This stimulus also reduces the metabolism of VIP by both the liver [7] and the lung [8], its major sites of degradation, thus ensuring that increased amounts of the peptide reach the kidney to increase sodium excretion. The demonstration by Morita and co-workers [3] that hepatic denervation abolishes the natriuresis following gastric sodium administration (as NaCl), together with our observations of changes in hepatic VIP metabolism [7], clearly suggest that intrahepatic events are important in the mediation system for the gastric sodium monitor.

In addition to the increase in the natriuretic peptide VIP, we and others have found that elements of the circulating renin–angiotensin system are affected by gastric sodium administration. In rabbits, gastric sodium administration, as NaCl, caused a rapid decrease in the plasma concentration of angiotensin II (ANG II), followed by an increase to a plateau level approximately half that of the basal ANG II concentration [9]. Thus a reduction in the sodium-conserving effects of the renin–angiotensin system may also contribute to the observed natriuresis. Further, we found that the change in the plasma ANG II concentration did not result from an increase in the rate of clearance of ANG II from the circulation, but from a decrease in the rate of synthesis or secretion of ANG II [9]. Measurements of plasma renin concentration showed no significant change in response to gastric sodium administration, although other workers have described a decrease in plasma renin activity [3] after this stimulus. As plasma renin activity reflects both renin concentration and angiotensinogen concentration, the two studies together suggest that a

change in angiotensinogen concentration may be one mechanism whereby there is a decrease in plasma ANG II.

Alternatively, it is possible that gastric sodium administration, as NaCl, may result in a decrease in the activity of angiotensin-converting enzyme (ACE) in one or more tissues, as pilot studies have indicated that a decrease in hepatic ACE activity occurs [10]. ACE is responsible for conversion of ANG I into ANG II in tissues as well as in the circulation, and has also been suggested by some workers to metabolize VIP [11]. In addition, it has been demonstrated that the natriuresis that ensues after gastric sodium administration can be prevented by prior inhibition of ACE [12]. Thus ACE may be important in mediation of the natriuresis.

We sought to determine, therefore, whether gastric sodium administration, as NaCl, might cause changes in the concentration of angiotensinogen in the plasma or in the liver. We also investigated whether changes in serum, hepatic or pulmonary ACE activity occur, thereby contributing to the observed decrease in the plasma ANG II concentration, the reduction in VIP metabolism and the increase in the plasma VIP concentration.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (n = 8 each group), weighing 250–300 g, were housed four to a box in a room with a 12-h light/dark cycle. The rats were permitted access *ad libitum* to a low (0.008%, w/w) sodium diet (Janos Chemicals, Forbes, NSW, Australia) and distilled water, and allowed to achieve sodium balance over a period of 7 days. In the 48 h prior to the experiment, the rats were placed in metabolic cages and urine was collected to determine urinary sodium excretion.

These studies complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990), and were approved by the Animal Ethics Committee of the University of New South Wales.

Circulating elements of the renin–angiotensin system

As our previous data relating to the effects of gastric sodium administration on renin and ANG II concentrations were obtained in the rabbit, we first determined whether similar results (a decrease in ANG II and no change in plasma renin concentration) were obtained with the rat.

On the day of experiment, the rats were anaesthetized using halothane (2.5%) in nitrous oxide (1 litre/min) and oxygen (0.5 litre/min) via a non-rebreathing mask. A 2 cm mid-line incision was made through the linea alba. After a 30-min rest equilibration period, the rats received a sodium load of 1.5 mmol/kg as 3× normal saline by direct gastric puncture using a 27 gauge needle. Aortic blood was sampled at 0, 15, 30 and 60 min (n = 8 in each group) after administration of the saline for measurement of plasma renin concentration, angiotensinogen concentration, ANG I concentration and ANG II concentration, and serum ACE activity. As the volume of blood required for these assays was 7–8 ml, four groups of eight rats (one group for each time point) were studied, so that volume-induced changes in renin and ANG II concentrations did not occur. For renin, angiotensinogen and ANG I, blood was collected into EDTA (0.05 ml/ml of blood) on ice and the separated plasma was stored at -20 °C until assay. For ANG II, blood was collected into pre-cooled syringes containing 0.3 M EDTA (0.05 ml/ml of blood) and 0.2 M 2,3-dimercapto-1-propanol (0.03 ml/ml of blood). After centrifugation, the plasma was extracted on a Sep-Pak C18 cartridge (Waters Corp., Milford, MA, U.S.A.) using 2 ml of acetonitrile/distilled water/acetic acid (74:24:4, by vol.). Specimens were then dried under nitrogen and stored at -20 °C until assay. For determination of ACE activity, blood was collected into a plain tube and allowed to clot. After centrifugation, the sera were stored at -20 °C until assay.

Hepatic angiotensinogen and ANG I concentrations

On the day of the experiment, the rats were anaesthetized as above, a mid-line incision was made through the linea alba and an iliac venous catheter was inserted. After a 30-min rest equilibration period, the rats were chosen at random to received a sodium load of 1.5 mmol/kg as 3× normal saline either intragastrically as above or via the iliac vein catheter. Livers were harvested at 0, 15, 30 and 60 min (n = 8 each group) and were immediately snap-frozen by immersion in liquid nitrogen before storage at -80 °C.

Hepatic and pulmonary ACE activity

Rats were anaesthetized and iliac venous catheters inserted as above. After a 30-min rest equilibration period, the rats received a saline load of 1.5 mmol/kg either intragastrically or intravenously. Livers were harvested at 0, 15, 30, 60 and 90 min after saline administration, and lungs were harvested at 0, 30, 60 and 90 min. Tissues were immediately snap-frozen by immersion in liquid nitrogen and stored at -80 °C until assay.

Laboratory assays

Plasma renin concentration

For determination of plasma renin concentration, 250 μl of plasma was incubated at 37 °C in the

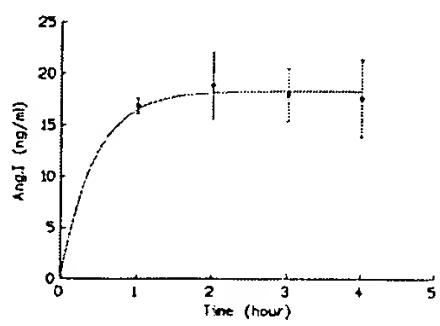
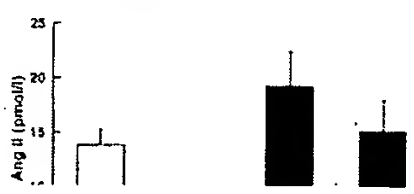


Figure 1 ANG I generation with increasing incubation time



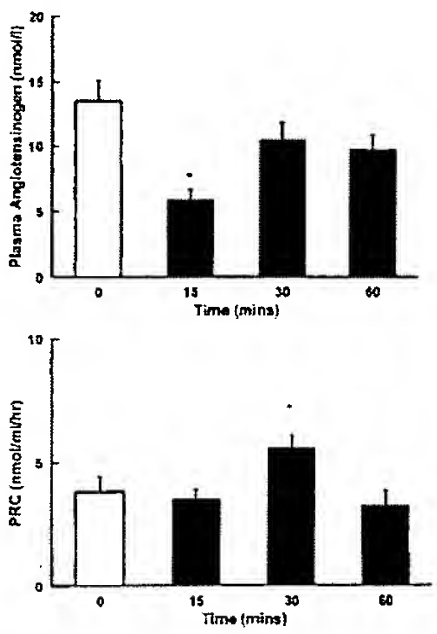
presence of an excess of renin substrate (prepared from nephrectomized rat plasma) for 2 h. The reaction mixture also contained 2,3-dimercaptopropanol (50 μl; 0.2 M) and PMSF (25μl; 8%, v/v) to inhibit angiotensinases. The ANG I generated was extracted on a C18 Sep-Pak using acetonitrile/distilled water/acetic acid (74:24:4, by vol.) and the eluate was blown down to dryness under nitrogen and stored at -20 °C until assayed. The extraction step was performed to remove the biologically inactive but immunologically reactive material present in plasma, as described by Waite et al. [13] and Menard and Catt [14]. Experiments showed that more than 95% of added ANG I was recovered through the Sep-Pak extraction. The ANG I was then determined by radioimmunoassay using a polyclonal antibody raised in rabbits (generously donated by Professor Graham Macdonald, Merck Sharpe & Dohme, Sydney, Australia), as described [15]. The polyclonal antibody used in this assay showed negligible cross-reactivity with ANG II or ANG III. The inter-assay coefficient of variation was 7%, and intra-assay coefficient of variation was 3.5%.

Plasma angiotensinogen concentration

To determine angiotensinogen concentration, 250 μl of plasma was incubated at 37 °C for 3 h in the presence of PMSF (25 μl; 8%, v/v) and 2,3-dimercaptopropanol (50μl; 0.2 M) to inhibit angiotensinases, and exogenous renin (10^{-5} units) was added (hog renin; Sigma). Experiments in which the ANG I generated in the reaction mixture was measured at serial time points showed that the concentration of ANG I generated had reached a plateau after 2 h (see Figure 1). This accorded with the work of Menard and Catt [14], Poulsen and Jorgensen [16], and Iwao et al. [17], who demonstrated substrate conversion to be complete in less than 2 h. To exclude the possibility that this plateau represented some degradation of generated ANG I, recovery experiments were performed. In these, exogenous ANG I was added to the plasma sample before incubation. The ANG I in paired 'spiked' and 'unspiked' incubates was extracted and measured by radioimmunoassay, as above. These experiments demonstrated no degradation of the added exogenous ANG I for incubation periods in excess of 4 h. This effectively excluded degradation of the generated ANG I as a reason for the plateau.

As with the assay for plasma renin concentration, the reaction product was extracted. This was done because studies which compared unextracted and extracted assays for angiotensinogen with bioassay showed a difference of up to 10-fold between the two methods [13,14]. This indicated that a significant amount of biologically inactive but immunologically reactive material was generated during the incubation step. For calculation of the angiotensinogen concentration, a zero incubation time blank, in which plasma was extracted before incubation, was subtracted. A zero time blank was included so that ANG I present in the plasma prior to incubation could be determined, as angiotensinogen concentration was to be estimated by the amount of ANG I generated during incubation. The values obtained lay within the normal range for angiotensinogen; this has been reported to vary from picomolar [18] to micromolar [17,19] concentrations, with a number of workers reporting values in the nanomolar range [13,14,20,21].

Figure 2 Plasma ANG II and ANG I concentrations after gastric NaCl administration in the rat



Plasma ANG II concentration

ANG II samples were reconstituted with barbitone buffer and assayed by radioimmunoassay, as previously described [9]. The polyclonal antibody used in this assay cross-reacts with ANG III, but has negligible cross-reactivity with ANG I under assay conditions. To determine the specificity of this assay system for ANG II, experiments were performed in which exogenous ANG I, ANG II or ANG III was added to plasma samples prior to Sep-Pak extraction. Less than 1% of the added ANG I at concentrations of up to 2500 pmol·l^{-1} was detected by the assay. Recovery of the exogenous ANG II exceeded 97%, while 90–95% of the added ANG III was detected in the assay. The intra-assay co-efficient of variation for this assay was 5%, and the inter-assay co-efficient of variation was 6%.

Serum ACE activity

Serum ACE activity was measured by a fluorimetric assay [22]. In brief, duplicate 10 μl serum aliquots were incubated with 240 μl of hippuryl-histidyl-leucine (5 mM) at 37 °C for 15 min. The enzymic reaction was terminated by addition of 1.45 ml of 0.28 M NaOH. The histidyl-leucine generated was estimated fluorimetrically following incubation with 2% *o*-phthaldialdehyde in methanol for 10 min. Fluorescence was determined by excitation/emission (350/500 nm) fluorimetry using an Hitachi F2000 fluorimeter. The amount of histidyl-leucine generated was derived by comparison with a standard curve.

Hepatic angiotensinogen and ANG I concentrations

For determination of hepatic angiotensinogen, 2–3 g of tissue was pulverized using a stainless steel hammer and anvil pre-cooled with liquid nitrogen. The pulverized tissue was placed in 20 ml of phosphosaline buffer (0.5 mol/l K_2HPO_4/1.5 mol/l NaCl, pH 8.4) containing perindoprilate (0.1 μg·ml^{-1}) (Servier Laboratories, Melbourne, Australia), EDTA (20 mmol·l^{-1}) (Ajax Chemicals, Sydney, Australia), 2,3-dimercaptopropanol (0.2 mol·l^{-1}) (Sigma) and 8% (v/v) PMSF (Sigma). The inhibitor mix was designed to prevent degradation of ANG I formed during the incubation step. The tissue homogenate was diluted 1:10 (w/v) with phosphosaline buffer, and duplicate 250 μl samples were incubated with 10^{-5} units·ml^{-1} renin (Sigma) for 4 h at 37 °C. Following incubation, the reaction mixture was diluted to 5 ml with 0.15 mmol·l^{-1} NaCl and extracted on a C18 Sep-Pak cartridge using 2 ml of acetonitrile/distilled water/acetic acid

Figure 3 Plasma angiotensinogen and renin concentrations following administration of a gastric sodium load in the rat

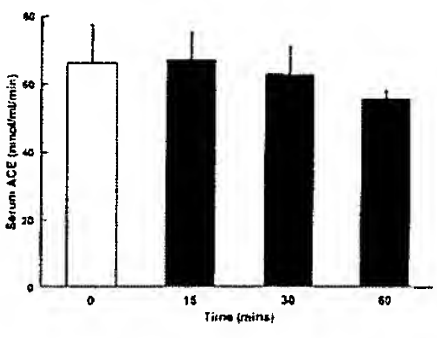


Figure 4 Serum ACE activity after gastric sodium loading

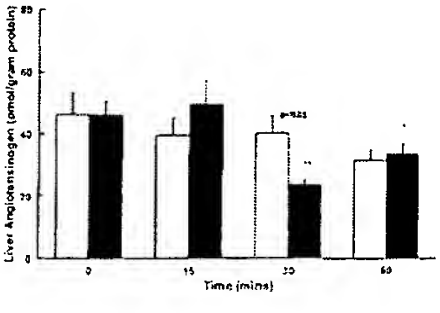


Figure 5 Hepatic angiotensinogen concentration after intravenous (open bars) and intragastric

(74:24:4, by vol.). The eluate was divided in two, and the duplicate samples were then blown down to dryness under a stream of nitrogen and stored at -20 °C until assay. The ANG I formed was measured by radioimmunoassay as above.

Recovery experiments were performed in which exogenous ANG I was added to the tissue homogenate before incubation. The experiments demonstrated that more than 75% of the added exogenous ANG I could be detected in the final assay mixture. To obtain the angiotensinogen concentration, each result was corrected for its zero incubation time blank (in which homogenate was extracted immediately), and then expressed as pmol/g of protein. The protein content of each homogenate was determined by the method of Lowry et al. [23].

For measurement of ANG I, the tissue was homogenized in phosphosaline buffer as above and immediately extracted on C18 Sep-Paks as above. After blowing down to dryness, ANG I was assayed by radioimmunoassay. The protein content of each homogenate was determined, and ANG I concentration was expressed as pmol/g of protein.

ACE activity in liver and lungs

Each organ was pulverized individually using a stainless steel tissue grinder and anvil pre-cooled with liquid nitrogen. The pulverized tissue was transferred into a centrifuge tube containing 20 ml of phosphosaline buffer, and homogenized using an Omni 2000 (Omni International) homogenizer at speed 3 for 60 s. The homogenate was then divided into aliquots and stored at -20 °C until ACE activity and protein assays were performed.

To determine ACE activity in tissue homogenates, variations in incubation time and dilution of homogenates were required. For lung, the homogenate was diluted to 1:100 and 1:400 with phosphosaline buffer, and duplicate 100 μl aliquots were then incubated with 240 μl of hippuryl-histidyl-leucine (5 mM) for 15 min; liver homogenates were incubated for 60 min. The reaction was stopped by addition of NaOH, and fluorescence was developed as above. Recovery experiments (in which the reaction product, histidyl-leucine, was included in the incubation mixture for each tissue) showed that no degradation of the product occurred during incubations of up to 45 min for lung and 60 min for liver.

The protein content of each tissue homogenate was determined by the method of Lowry et al. [23]. Tissue ACE activity was expressed as nmol of histidyl-leucine generated·min^{-1}·g^{-1} protein for liver, and as μmol of histidyl-leucine generated·min^{-1}·g^{-1} protein for lung.

Statistical methods

Changes in measured parameters with time were assessed using analysis of variance. When significant differences were found, individual comparisons were made by *t*-test using a pooled variance estimate (CSS; Statistica). Comparisons at each time point between intragastric and intravenous groups were carried out by *t*-test using a pooled variance estimate. *P* values of < 0.05 were accepted as significant.

RESULTS

Plasma ANG II, renin and angiotensinogen concentrations

Plasma ANG II levels decreased significantly after gastric sodium administration. At 15 min, it had decreased from the control value of 13.9±1.4 pmol·l^{-1} to 7.4±0.6 pmol·l^{-1} ($P < 0.005$). Thereafter, plasma ANG II returned to baseline (Figure 2, upper panel). Plasma ANG I also decreased significantly in response to gastric sodium administration: it had decreased from 1.38±0.1 nmol·l^{-1} in the control group to 0.6±0.1 nmol·l^{-1} ($P < 0.05$) by 15 min after sodium administration (Figure 2, lower panel). Thereafter, plasma ANG I increased non-significantly, but remained below control values.

The plasma renin concentration was unchanged at 15 min after administration of the gastric sodium load (Figure 3, upper panel), but had increased significantly at 30 min ($P < 0.05$). However, plasma angiotensinogen had decreased significantly from the control value of 13.6±1.6 nmol·l^{-1} to 5.9±0.8 nmol·l^{-1} ($P < 0.005$) at 15 min after administration of the saline (Figure 3, lower panel). Thereafter, plasma angiotensinogen returned to baseline. Serum ACE activity was unchanged after gastric sodium compared with the control value (Figure 4).

Angiotensinogen and ANG I concentrations in the liver

The angiotensinogen concentration in the liver decreased after gastric sodium administration, but not after intravenous sodium administration (Figure 5). In the rats which received an intragastric saline load, no decrease from the control level was evident at 15 min. However, at 30 min hepatic angiotensinogen had decreased from 46.0±4.4 pmol·g^{-1} protein in the control group to 23.8±1.4 pmol·g^{-1} protein ($P < 0.005$). The hepatic angiotensinogen concentration 30 min after gastric saline administration was also significantly lower than that after intravenous saline ($P < 0.05$). This reduction in hepatic angiotensinogen concentration after intragastric saline was maintained at 60 min (33.7±3.4 pmol·g^{-1} protein; $P < 0.01$) compared with the control.

(shaded bars) sodium loading

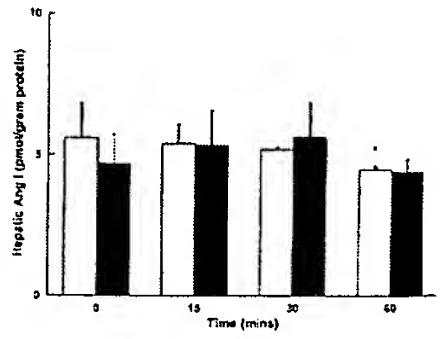


Figure 6 Hepatic ANG I concentration after intravenous (open bars) and intragastric (shaded bars) sodium loading

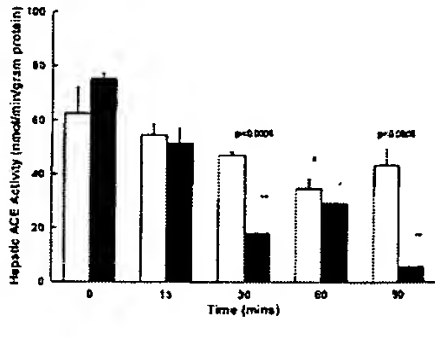


Figure 7 Hepatic ACE activity after intravenous (open bars) and intragastric (shaded bars) sodium loading

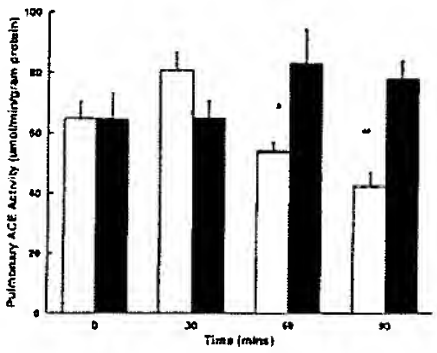


Figure 8 Pulmonary ACE activity after intravenous (open bars) and intragastric (shaded bars) sodium administration

In contrast with angiotensinogen, the hepatic concentration of ANG I was unchanged after gastric sodium administration, but decreased in response to intravenous saline administration (Figure 6).

ACE activity in the liver and lungs

In the liver, ACE activity decreased after both intragastric and intravenous sodium administration, although the time frame for the decrease differed between the two routes of administration (Figure 7). After the intragastric saline load, ACE activity had decreased significantly from the control level of 75.4±17.8 nmol·min^{-1}·g^{-1} protein to 18.0±2.0 nmol·min^{-1}·g^{-1} at 30 min ($P < 0.005$). Hepatic ACE activity then remained significantly lower than control at both 60 and 90 min ($P < 0.01$ and $P < 0.005$ respectively). There were significant differences between the intravenous and intragastric groups at 30 min ($P < 0.0005$) and 60 min ($P < 0.0005$).

After intravenous sodium administration, hepatic ACE activity was decreased at 60 min ($P < 0.005$) and then returned towards baseline (Figure 7).

In the lungs, no significant change in ACE activity occurred after gastric sodium administration. However, intravenous saline caused significant decreases in ACE activity at 60 and 90 min (Figure 8).

DISCUSSION

The present study shows that the decrease in circulating ANG II levels that we observed in the rabbit after administration of a gastric sodium load also occurs in the rat. Further, it confirms that the plasma renin concentration is unchanged in the rat after gastric sodium loading, as was the case in the rabbit. Although we have found in both the rat and the rabbit that the plasma renin concentration is unaffected by gastric sodium loading, Morita and co-workers [3] demonstrated that plasma renin activity decreased. As plasma renin activity reflects the concentrations of both renin and angiotensinogen, the decrease observed by Morita et al. [3] may be explained by changes in the angiotensinogen concentration. Our data in the rat support this hypothesis. Serum and pulmonary ACE activities were unchanged after gastric sodium administration. These data effectively exclude the possibility that a change in the activity of this enzyme in lung or serum contributed to the decrease in ANG II. Instead, there were decreases in the plasma concentrations of ANG I and angiotensinogen 15 min after administration of the gastric sodium load. As the plasma renin concentration was unchanged, the decreases in ANG I and ANG II appear to result from the decrease in plasma angiotensinogen levels. This decrease in circulating angiotensinogen may be attributable to a decrease in its secretion rather than to a decrease in synthesis, as hepatic angiotensinogen did not decrease until 30 min after the gastric sodium load had been administered.

The time course of the decrease in plasma ANG II concentration differed in the rat and the rabbit. In the rat, plasma ANG II levels remained depressed for less than 30 min, while in the rabbit the decrease in circulating ANG II persisted for 1–2 h [9]. The more prolonged response in the rabbit may reflect species differences or, alternatively, may be due to an effect of anaesthesia, as the experiments in rabbits were performed on conscious animals. Despite the short duration of the plasma response, there were prolonged changes in the intrahepatic renin–angiotensin system in the rat in response to administration of the saline solution intragastrically. These changes are a response to stimulation of the gastric sodium monitor, as administration of the same saline load intravenously evoked either no response (hepatic angiotensinogen) or a delayed response of shorter duration (hepatic ACE).

Synthesis of angiotensinogen by the liver is known to be controlled by ANG II in a positive-feedback loop [21,24], with higher ANG II concentrations resulting in increased angiotensinogen synthesis. This increase in angiotensinogen synthesis is achieved because ANG II stabilizes the mRNA for angiotensinogen [25,26]. The decrease in hepatic angiotensinogen concentration 30 min after administration of the gastric sodium load would appear, therefore, to occur in response to the earlier decrease in the plasma ANG II concentration. Alternatively, the decrease in hepatic angiotensinogen may be secondary to the decrease in ACE activity in the liver, as treatment with ACE inhibitors has been found to decrease angiotensinogen secretion and synthesis [27,28]. This second possibility would seem less likely, as we found that both angiotensinogen concentration and ACE activity in the liver decreased at the same time point. A third possibility is that the decrease in angiotensinogen synthesis occurred in response to a change in hepatic nerve activity. Morita and co-workers [3] demonstrated that gastric sodium loading decreased plasma renin activity, and that this decrease could be prevented by hepatic denervation [3]. Our data provide a mechanism whereby an alteration in hepatic nerve activity engenders a decrease in plasma renin activity.

Thus we conclude that changes in angiotensinogen secretion and synthesis and ACE activity in the liver contribute to the mediation system for the gastric sodium monitor.

ACKNOWLEDGMENTS

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